Prostaglandin E2 amplifies epidermal growth factor-induced phosphorylation of extracellular signal-regulated kinase (ERK) in hepatocytes

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kull V-01 våren 2006

Prosjektoppgave for Medisin (profesjonsstudium)

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ACKNOWLEDGEMENTS

The present study was carried out at the Department of Pharmacology, the Faculty of Medicine, University of Oslo. I have received funding from the Medical Student Research Programme at the University of Oslo (Incentive Funding) and from the Norwegian Cancer Society while doing research.

I would like to thank my tutor Thoralf Christoffersen, and Olav Dajani, Kristin Meisdalen, Eva Østby, Monica Aasrum, Tormod Guren, Dagny Sandnes and Hege Thoresen whom I have been cooperating with for this study. A special thanks goes to Mina Lam for her good companionship and for taking an interest in the smallest details concerning my research.

Oslo, April 2006

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ABSTRACT

The signalling pathways of G-protein coupled receptors (GPCRs) and epidermal growth factor receptors (EGFRs) have been shown to communicate in mitogenic processes in many cells. Several mechanisms have been suggested to explain this; in particular transactivation is a mechanism that seems to be involved in many cases where signalling pathways intermingle. We have observed that prostaglandin E2 (PGE2) and other GPCR ligands enhance the mitogenic effect of epidermal growth factor (EGF) on hepatocytes. In this study we suggest that cross-talk between the pathways activated by these two agonists is not dependent on transactivation but a result of altered gene transcription caused by stimulation with PGE2. Our results indicate that transcription is altered so as to induce proteins that prolongate signalling in the EGFR pathway. These proteins inhibit the deactivation of the extracellular-regulated kinase (ERK), which is an important component in the pathway dowstream of the EGFR.

BACKGROUND

Multicellular organisms depend on keeping a proper balance between the proliferation, growth and death of their cells. In order to keep this balance the cells need to communicate with each

other. Messages from one cell to another are mediated by molecules (mostly proteins) they secrete or have bound to their surface. Some of these molecules are growth factors and mitogens that induce growth and proliferation, others are survival factors that suppress apoptosis. All cells need to pass certain check points in their cell cycle in order to proliferate, and without the "permission" of a signal from a growth factor most cells are unable to get beyond these check points. When the balance between these growth regulating signals is disturbed the result may be excessive proliferation or a shortage of cell death, and in the end lead to proliferative disorders such as cancer.

In this study we focus on the effect of a certain growth factor – the epidermal growth factor (EGF) – and its interaction with a prostaglandin (PGE2) which is known to enhance proliferation in many cells. We have observed that PGE2 enhances the proliferative effect of EGF in hepatocytes, and in this study we suggest that PGE2 does so by altering gene transcription and thereby modulating components in the intracellular pathway activated by EGF.

The EGF receptor

EGF mediates its signal by binding to the epidermal growth factor receptor (EGFR) on the cell surface. The EGFR is a membrane bound receptor, and one of its main functions is the regulation of cellular proliferation. Based on receptor affinity and structure the EGFR and three other receptors form a subfamily known as the ErbB receptor family (Holbro and Hynes, 2004). This subfamily belongs to the larger superfamily of receptor tyrosine kinases (RTKs) which all have certain qualities in common: They have a single transmembrane segment, and their intracellular

part contains a kinase domain that specifically phosphorylates tyrosine residues in other proteins. The intracellular kinase domain is activated upon binding of ligands (such as EGF) to the extracellular part of the receptor (Alberts et al., 2002).

The EGFR is strongly involved in the control of cellular proliferation. This is suggested not only by in vitro experiments, but by the fact that many epithelial cancers are known to overexpress the EGFR or its ligands, or to contain mutated variants of the receptor (Jorissen et al., 2003). It has been difficult to pin down the physiological role of the EGFR because rodents with null mutations in the ErbB signalling system have died in utero or barely survived a few weeks after birth. In EGFR-null mice the organs particularly affected are skin, liver, gastrointestinal tract, lungs and brain (Jorisson et al., 2003; Holbro and Hynes, 2004). These findings confirm previous studies that have described EGFR as a regulator of proliferation.

Activation of the EGFR

Six ligands have been described for the EGFR. The epidermal growth factor (EGF) and the transforming growth factor alpha (TGFα) are the ones that have been most extensively studied; in addition amphiregulin, betacellulin, heparin-binding EGF and epiregulin may also activate the receptor (Holbro and Hynes, 2004). Without ligand stimulation there is an equilibrium of dimers and monomers of EGFR molecules in the surface of the cell (Jorissen et al., 2003). The dimers can be either homodimers or heterodimers, i.e. they may contain either two identical or two different members of the ErbB subfamily. The binding of a ligand to the ectodomain of such a

dimer causes a conformational change in the intracellular part of the receptor. This reorganisation brings the katalytic tyrosine kinase domains on each of the two receptor molecules into a position where they can phosphorylate and activate each other and also (auto- and trans-) phosporylate tyrosine residues on the non-katalytic parts of the two receptor molecules (Jorissen et al, 2003). Some cytoplasmic proteins contain highly conserved domains that specifically recognise these phosphorylated tyrosines. They are known as SH2 (src homology 2) domains and PTB (phosphotyrosine-binding) domains. Cytoplasmic signalling proteins can dock on to the EGFR via these domains. Proteins that do not contain SH2 or PTB domains may dock on to the EGFR by means of adaptor proteins containing SH2 domains. The cytoplasmic proteins that have associated with the activated receptor are themselves activated in different ways, most likely either by phosphorylation of a tyrosine residue, by allosteric activation or by translocation to the membrane (where they are located in proximity to activating molecules). Further signalling proteins contain additional specific domains (such as SH3 domains and pleckstrin homology (PH) domains) that recognise certain sequences (such as proline rich sequences and phosphoinositide lipids, respectively) in receptor bound proteins and proteins further downstream (Schlessinger, 2000). This allows for the extracellular signal to be transduced through the intracellular machinery in a way that is specific and not arbitrary.

The Ras/MAPK signalling pathway

Several different signaling pathways have the EGFR as a common point of origin. One of them is the pathway leading to the activation of the mitogen-activated protein kinases (MAPKs) via Ras. In this signalling cascade the adaptor protein Grb2 links up with the EGFR directly via its SH2

domain, or indirectly by means of another adaptor protein, Shc, that is tyrosin phosporylated by the EGFR and subsequently associates with Grb2. Grb2 contains a SH3 domain by which it is constitutively associated with Sos which acts as a GDP-GTP exchange factor. When the Grb2-Sos complex has docked on to the EGFR it is translocated to the plasma membrane where it activates Ras (Schlessinger, 2000). Ras is a family of small membrane bound G-proteins that are activated when its GDP is exchanged for GTP. Ras genes are mutated and expressed as an overactive variant in about one in four human tumours. Not surprisingly, several pathways with point of departure in Ras have been subject to detailed scrutiny (Alberts et al., 2002).

In the Ras/MAPK pathway Ras interacts with a signalling casette of kinases that are highly conserved in evolution. At the end of this cascade are the mitogen-activated protein kinases (MAPKs). This is a group of enzymes that convey information from cell surface receptors to intracellular targets that regulate proliferation, cell death and survival, differentiation and migration. Common to these kinases is that they are activated by the phosphorylation of both a threonine and a tyrosine residue within each of them (Alberts et al., 2002). The MAPKs are activated by the upstream MAPK kinases (MAPKK), which in turn are activated by MAPK kinase kinases (MAPKKK), and these again are activated by Ras. Each MAPK is specifically activated by only one of the immediate upstream MAPKK. The MAPKKs, however, can be activated by a number of different MAPKKKs. This enhances the complexity and diversity of the signalling system (Chang and Karin, 2001).

Some of the activated MAPKs are translocated to the nucleus where they regulate transcription factors. Others remain in the cytoplasm and regulate gene expression by means of post transcriptional mechanisms. As to the biological outcome MAPKs are involved in the regulation

of almost all cellular processes, but most well known are their functions as regulators of proliferation and cell survival (Chang and Karin, 2001).

The role of ERK

The MAPK of particular interest to this study is the extra cellular signal-regulated kinase 1 and 2 (ERK 1/2). ERK 1/2 is activated specifically by MEK 1/2 (a MAPKK), which in turn is activated by Raf-1 (a MAPKKK), and Raf-1 is activated by Ras. When ERK is phosphorylated it translocates to the nucleus where it activates the Ets transcription factors. Ets in turn promotes the synthesis of c-Fos, which associates with members of the jun family to form AP-1 (activator protein 1) (Chang and Karin, 2001). This dimer binds to the promotor of several genes, the one for cyclin D1 being one of them (Cook et al., 1999). Cyclins control the progression through the cell cycle and cyclin D1 is active in the G1 phase of the cell cycle. It is part of the cyclin dependent protein kinase Cdk4/6 which phosphorylates the Retinoblastoma tumour suppressor (Rb), thus releasing the transcription factor E2F and enabling it to induce the synthesis of further cyclins (E and A) (Ussar and Voss, 2004; Milde-Langosch 2005). In other words, ERK triggers cell cycle progression by promoting the production of certain cyclins at beginning of the cycle. Upon stimulation with EGF, ERK also stimulates synthesis of pyrimidine nucleotides, by phosphorylating carbamoyl phosphate synthetase II, which is a rate limiting enzyme for this process (Wilkinson and Millar, 2000; Chang and Karin, 2001).

ERK is also involved in regulation of cell cycle progression by means of posttranslational mechanisms. When ERK activation is sustained, ERK phosphorylates c-Fos and contributes to

the stability of this protein (Murphy et al., 2002; Milde-Langosch 2005). Furthermore, the construction of cyclinA- and cyclinE-Cdk2 kinase complexes has been linked to the presence of the p21 and p27 Cdk inhibitor proteins (CKIs) which function as assembly factors in this context. ERK increases the number of CDKs available for this purpose (Wilkinson and Millar, 2000).

However, the role of ERK is not restricted to the beginning of the cell cycle, and neither is the nucleus its sole area of operation. Recovery after radiation induced arrest in cell cycle at the G2/M check point has been shown to be dependent on ERK (Wilkinson and Millar, 2000). It has also been suggested that the natural arrest of unfertilised oocytes at metaphase in meiosis II is induced by a cytostatic factor that is in need of ERK for its activity (Chang and Karin, 2001; Gross et al., 1999). A part of the pool of activated ERK does not translocate to the nucleus but remains in the cytoplasm. ERK in this position takes part in the translation of mRNA into protein by phosphorylating the eukaryotic translation factor-4E (eIF-4E) (Wilkinson and Miller, 2000; Chang and Karin, 2001).

The PI3K pathway

Another important pathway regulating proliferation is the phoshpoinositide 3-OH kinase (PI3K) pathway. Like the Ras/MAPK pathway this signalling cascade is activated by RTKs. PI3K may be activated by binding directly to the RTK with its regulatory subunit p85, but activation may also be dependent on Grb2 and Ras (Cully et al., 2006). The EGFR is one of the RTKs that activate PI3K. Activation of the PI3K pathway works in the same direction as the Ras/MAPK

pathway by inhibiting apoptosis. It is one of the most important anti-apoptotic pathway in cells, and its activity is enhanced in many tumours (Downward, 2004).

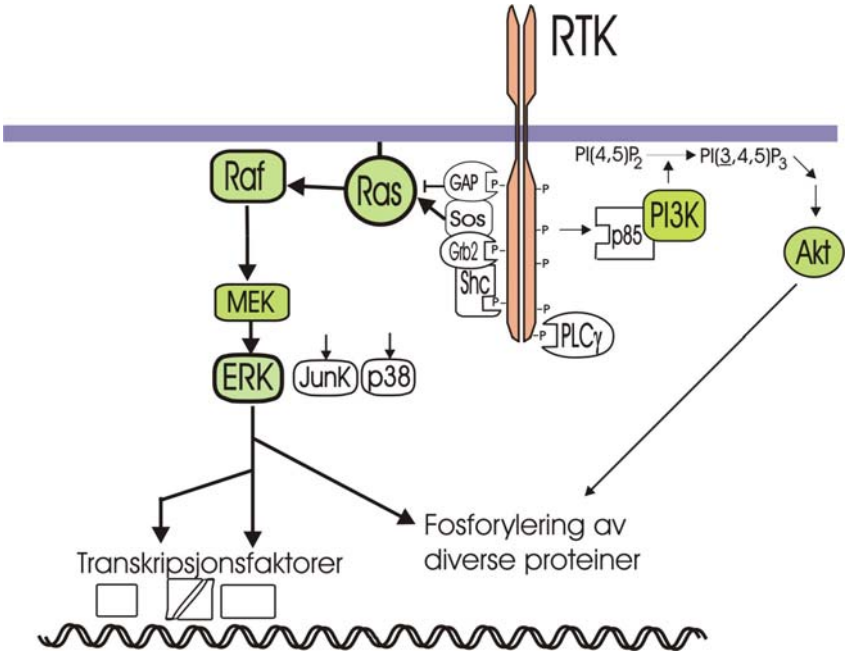

Figure 1 The signalling pathway from the EGFR (RTK). The figure was borrowed from other members of our group.

Specificity

How are the numerous signalling molecules organised into separate pathways? The specificity in the interaction between signalling proteins is provided for by the fact that they possess specific amino acid sequences that recognise corresponding sequences in other proteins, as illustrated in the signalling pathway described above. A few other ways of avoiding random combination have been described. Certain pathways utilise scaffold proteins in their signal transduction. These are

proteins that assemble interacting signalling molecules and isolate them from other closely related pathways. Another sorting mechanism is the selective spatial localisation of proteins, such as translocation to the membrane or the nucleus (illustrated above), or colocalisation to the cytoskeleton (Schlessinger 2000, Chang and Karin, 2001). Specificity also means that the same signalling molecule or receptor can have various effects in different sort of cells – and also in cells of one and the same kind. This is most likely due to the fact that different cells will have a different repertory of effector proteins that interact with the same kind of signalling molecule. Cells of the same cell lineage may respond differently to the same extracellular signal because they have reached different stages of differentiation. Also the duration and intensity of a signal may be decisive for the biologic outcome it is going to have (Wilkinson and Miller, 2000).

Attenuation of signal

A number of mechanisms are involved in the negative regulation of signalling in the EGFR pathway. Dephosphorylation of activated proteins, downregulation of receptors by endocytosis and breakdown of proteins in proteasomes are some of the processes involved, often regulated by negative feedback in the pathway. Of particular interest to this study is the deactivation ERK. ERK is dephosphorylated by the dual specificity phosphatases MKP1/2 and PAC1, which are able to dephosphorylate phosphothreonine and phosphotyrosine in ERK (Hunter, 1995). These phosphatases are nuclear proteins that are expressed transiently in response to ERK activation, and they act specifically on MAPKs (Alessi, 1995). Removal of one of the phosphates in activated ERK is sufficient to deactivate the protein, thus the serine-threonine specific non-receptor protein phosphatase PP2A is also able to deactivate ERK (Alberts, 2002). PP2A has

been associated with the inactivation of ERK that takes place within minutes after stimulation with growth factor, while the MKPs are associated with the later inactivation that is assumed to depend on protein synthesis (Pouysségur and Lenormand, 2003). However, the exact role of each of the phosphatases varies among different cell lines and the knowledge about the phosphatases is far from complete.

Prostaglandins

Besides signalling from the EGFR prostaglandins have proved to be another important factor in the regulation of cellular proliferation. The link between prostaglandins and cancer has been extensively studied, particularly after it was discovered that NSAIDs reduce the risk of colorectal cancer. Prostaglandins are derivatives of arachidonic acid, and COX is the enzyme that converts arachidonic acid to prostaglandins. The prostaglandin receptors belong to the group of heterotrimeric G-protein coupled receptors (GPCRs). One of the best studied prostaglandins is prostaglandin E2 (PGE2). This agonist activates different subtypes of prostaglandin receptors, and in turn it therefore also activates different G-proteins depending on the subtype of receptor that is stimulated. Both Gq, Gs and Gi can participate in PGE2 induced signalling (Bos et al., 2004). Numerous studies have shown that PGE2 is involved in the promotion of tumour growth (Wang and DuBois, 2006).

Cross-talk among different signalling pathways

Traditionally it has been assumed that most signalling pathways operate in separate signalling cassettes that have little communication with each other. However, it has become evident that a signal can involve several different pathways on its route through the cell. Signals from GPCRs and the EGFR have been proved to intermingle, and the cross-talk between these pathways has been extensively studied. Another pathway that has been suggested to be involved in mediating between GPCR stimulation and ERK activation is signalling via integrin-based focal adhesions. It has also been reported that the GPCR itself may act as a scaffold for complex assembly that ultimately leads to the activation of ERK (Luttrell et al., 1999). The contribution from each of these models varies not only among different sorts of cells but also among cells of the same kind (Pierce et al., 2001).

The interaction between these pathways has been studied by means of different kinds of experiments. Typically, these experiments have looked for the GPCR ligand-induced activation of signalling proteins or receptors at (or below) the point where the two signalling pathways are assumed to converge (such as phosphorylation of the EGFR or Shc). In other experiments the signalling molecules at (or upstream of) the point of convergence have been inhibited in order to see whether the pathway below is affected by this. Instead of using a pharmacological inhibitor the same kind of information can be obtained by transfecting cells with a mutated and dysfunctional receptor or signalling protein. It can also be demonstrated that proteins from different signalling pathways form complexes by showing that they coprecipitate in experiments (Luttrell et al., 1999).

Taking a closer look at the pathway below the EGFR it is evident that a signal from a GPCR can intervene at several stages in this pathway. The EGFR can be transactivated in a direct or an indirect manner. In some cases the signal passes from the GPCR intracellularly (and directly) to let a non-receptor tyrosine kinase (such as c-src) activate the EGFR (Eguchi et al., 1998). In other cases the signal passes the membrane two more times before the receptor is activated. That is, the GPCR may induce the production of EGFR ligands that activate the receptor in an autocrine way, or it may cause the release of EGFR ligands that are bound to the surface of the cell as ligand precursors (Prenzel et al., 1999). It has also been shown that COX-2 may induce the synthesis of EGFRs (Wang and DuBois, 2006). However, as mentioned above, the EGFR need not be involved in the cross-talk at all. Integration of the pathways may take place at the level of Ras, or even lower, as Ras-independent activation of ERK through GPCR-stimulation has been described (Dhanasekharan et al., 1998).

In these models signalling in the EGFR and Ras/MAPK pathways are modulated by signalling from GPCR. However, cross-talk may also occur the other way around. Thus, in some cells increased expression of COX-2 and PGE2 is a downstream result of stimulation of the EGFR (Coffey et al., 1997; Chien et al., 2006; Pai et al., 2006). Hepatocytes are no exception to this. Both EGF and TGFα have proved to cause the release of prostaglandins in hepatocytes. Prostaglandins, on the other hand, have the ability to amplify the proliferative effect of these growth factors (Christoffersen et al., 2000). These models do not necessarily contradict each other. They may be part of an amplifying loop.

Cross-talk in hepatocytes

PGE2, PGF2α, angiotensin II, vasopressin, glucagon and norepinephrine are all GPCR-agonists that reinforce the proliferative effect of EGF in hepatocytes (Refsnes et al., 1994; Christoffersen et al., 2000). They are referred to as comitogens because they enhance the effect of mitogens such as EGF rather than initiating DNA-synthesis on their own. However, the distinction between mitogens and comitogens is not absolute, as some comitogens have proved to be able to induce proliferation on their own. The response to comitogenic stimuli in hepatocytes is greatest early in the G1 phase of the cell cycle (e.g. 0-10 h after plating), whereas the effect of mitogens is greatest when they are added in mid/late G1 (40-50 h). It is assumed that the comitogenic agonists accelerate the traverse through the early G1 phase of the cell cycle, thus increasing the pool of cells that is responsive to mitogens later in G1. However, the exact mechanism for the comitogenic effect is not known (Christoffersen et al., 2000; Nilssen et al., 2002).

Our project

In the present study we have looked at the mechanisms for the interaction of EGF and PGE2 in hepatocytes. In other cells PGE2 has been shown to transphosphorylate the EGFR, and downstream events such as DNA-synthesis and activation of ERK have proved to be dependent on this transphosphorylation (Pai et al., 2002). In hepatocytes, however, the comitogenic effect of a number of GPCR agonist, including the prostaglandin PGF2α has been demonstrated not to be dependent on EGFR transphosphorylation (Nilssen et al., 2004). In this study we suggest that in

hepatocytes the synergism between GPCR agonists and EGFR is mediated neither by transactivation nor by any of the other mechanisms mentioned above.

We have chosen the activation of ERK as one of the "end points" in this study. It is known that the proliferative effect of PGE2 is dependent on ERK-activation in certain cells (Pozzi et al., 2004). The mitogenic effect of EGF in hepatocytes is also dependent on the (sustained) activation of ERK (Thoresen et al., 2003). For these reasons it seems adequate to investigate how ERK is affected by PGE2 and EGF in hepatocytes. However, it has been shown that comitogenic signalling in hepatocytes is not dependent on the activation of ERK in early G1 (when hepatocytes are most sensitive to comitogens) (Nilssen et al., 2002). Stimulation of hepatocytes with PGF2α and some other GPCR agonists in combination with EGF have shown that ERK may well be inhibited in early G1 without curbing the comitogenic effect (Nilssen et al., 2002). PGE2, however, is a prostaglandin whose effect on ERK in hepatocytes in not known.

MATERIALS AND METHODS

Materials

MH1C1 cells were from American Type Culture Collection. Dubecco's modified Eagle's medium, Dulbecco's phosphate buffered saline, HEPES, penicillin, streptomycin and horse serum were from Gibco (Grand Island, NY). William's medium E was from Whittaker

(Walkerville, MD). Collagenase (C-0130), dexamethasone, free bovine serum albumin (BSA) (fraction V), collagen (type I from rat tail), actinomycin, natriumorthovanadat and EGF (from mouse) were from Sigma Chemical Co. (St. Louis, MO). AG1478 and cycloheximide were from Calbiochem (La Jolla, CA). Penstrep was from Cambrex Bio Sciences (Verviers, Belgium). Gefitinib was a gift from Astra Zeneca.

Cell isolation and culture

Male Wistar rats, 170-230 g, were fed ad libitum. Hepatocytes were isolated by a two-step in vitro version (Seglen, 1976) of the collagenase perfusion technique (Berry and Friend, 1969) with modifications as previously described (Christofferesen et al., 1984). Hepatocytes as well as MH1C1 cells were seeded onto Costar plastic culture wells or flasks, at a cell density of 2 x $10^4/cm^2$. The culture medium used for hepatocytes was a serum-free 1:1 combination of William's medium E and Dulbecco's modified Eagle's medium (with final glucose concentration 8.4 mM). The medium was supplemented with penicillin (67 μg/ml), streptomycin (100 μg/ml) insulin (100nM), and dexamethasone (25nM). The culture medium used for MH1C1 was Dulbecco's modified Eagle's medium. The medium was supplemented with horse serum (10%) (apart from the last 24 hours before stimulation), penicillin and streptocmycin (Pen Strep 100 units/ml). All cell cultures were kept in 95% air /5% CO_2 a 37°C.

Immunoblotting

Aliquots with 20 μg cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed in 12% (w/v) polyacrylamide gels (acrylamide:N'N'-bis-methylene acrylamide 29:1) followed by immunoblotting with a phospho-specific MAPK antibody detecting p44mapk and p42mapk (ERK1 and 2) only when catalytically activated by phosphorylation at Tyr204 or Thr202/Tyr204 (New England Biolabs, Inc., Beverly, MA.). Immunoreactive bands were visualised with Enzyme-linked Chemiluminescence (ECL).

RESULTS AND DISCUSSION

Preparatory experiments

Preparatory experiments were done in order to find adequate concentration and treatment time for the agonists and inhibitors used in our experiments, and in order to find typical responses of ERK in different cells upon stimulation with agonists. Data from some typical experiments with the MH1C1 cells are shown in fig. 1. Hepatocytes were investigated by other members of our group. Interestingly, the MH1C1 cells proved to be more sensitive to the tyrosine kinase inhibitor gefitinib than the hepatocytes, requiring lower doses for complete inhibition of downstream activation of ERK.

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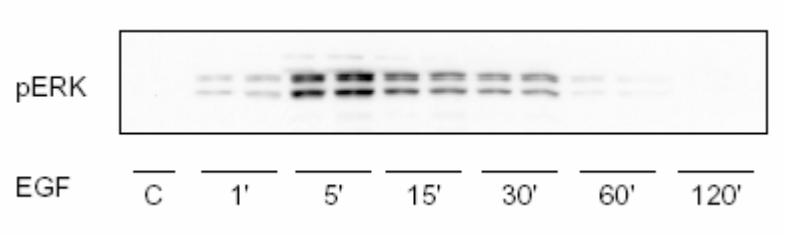


Figure 2a Time course for EGF in MH1C1 cells. ERK response was measured in MH1C1 cells incubated with EGF (10 nM) for time periods of 1 min to 48 h. Phosphorylation of ERK reached a maximum after 5 min, thereafter declining gradually over 3 h.

2b.

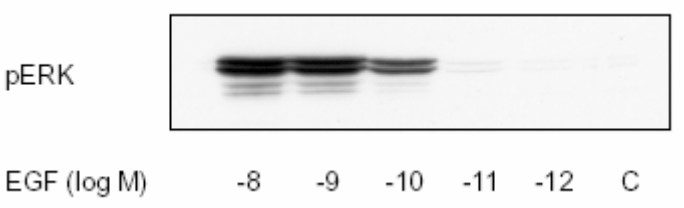


Figure 2b Dose-response curve for EGF-induced ERK activation in MH1C1 cells. MH1C1 cells were stimulated with EGF in various concentrations for 5 min. The results show that 1 to 10 nM EGF is an appropriate concentration for further experiments with EGF.

2c.

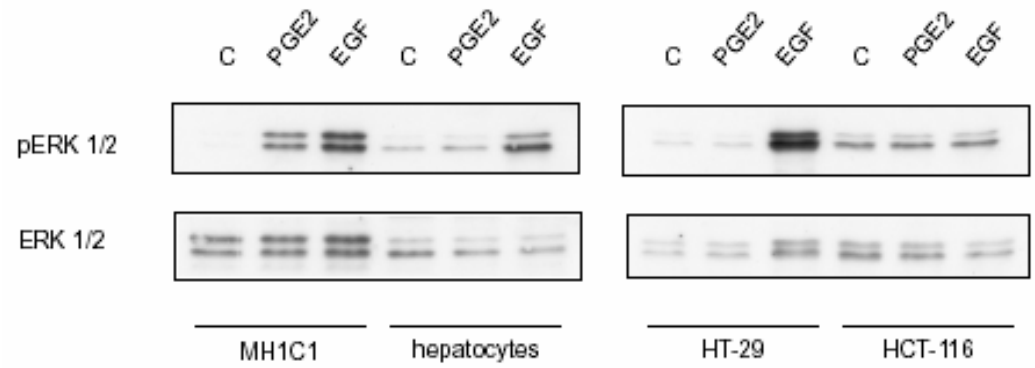


Figure 2c ERK response in various cell types to stimulation with EGF (10 nM) and PGE2 (100 μM) for 5 min. HT-29 and HCT-116 are human colon cancer cell lines. In HCT-116 cells ERK is constitutively activated. In all other cells ERK is phosphorylated upon stimulation with EGF, while only MH1C1 cells show activation of ERK in response to PGE2. Normally ERK would be activated in hepatocytes as well after PGE2 stimulation for 5 min, but the results are not always reproducible.

2d.

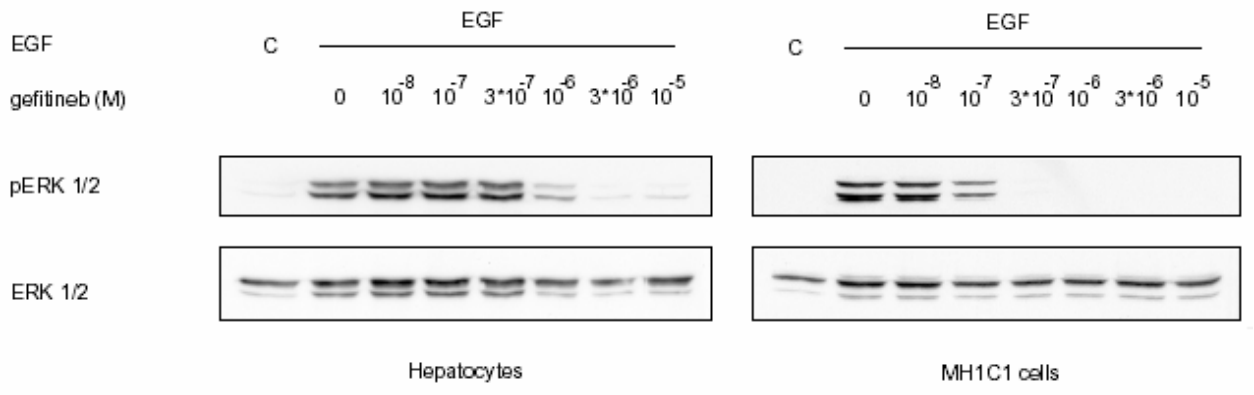


Figure 2d Dose-response curve for getifinib in hepatocytes and MH1C1 cells. The cells were stimulated with EGF for 5 min after 3 h in culture, and gefitinib was added 30 min before stimulation with EGF. MH1C1 cells are more sensitive to gefitinib than hepatocytes are. MH1C1 cells are completely inhibited at $3*10^{-7}$ M gefitinib, while this concentration has no inhibitory effect in hepatocytes, which need $3*10^{-6}$ for complete inhibition.

Transphosphorylation of the EGFR is necessary for the PGE2 induced activation of ERK in MH1C1 cells, but not in hepatocytes.

As transphosphorylation of the EGFR is assumed to explain cross-talk with GPCRs in many cell lines, but not in hepatocytes, we wanted to find out whether transactivation was necessary for the PGE2-induced activation of ERK in hepatocytes and MH1C1 cells. Various studies have concluded that transactivation is a rapid process (Prenzel et al., 1999), and for this reason the cells were incubated with PGE2 or EGF for only 5 minutes. For inhibition of the EGFR we used gefitinib and AG1478 which both specifically inhibit the tyrosine kinase of the EGFR. The results (fig. 3) show that gefitinib inhibits PGE2 induced activation of ERK in MH1C1 cells, but not in hepatocytes. Similar results are shown for AG1478 in hepatocytes. This indicates that

MH1C1 cells are dependent on transactivation of the EGFR for the phosphorylation of ERK, whereas hepatocytes use other mechanisms for ERK activation. The complete absence of phosphorylated ERK in cells treated with EGF and gefitinib or AG1478 confirms that these inhibitors are effective.

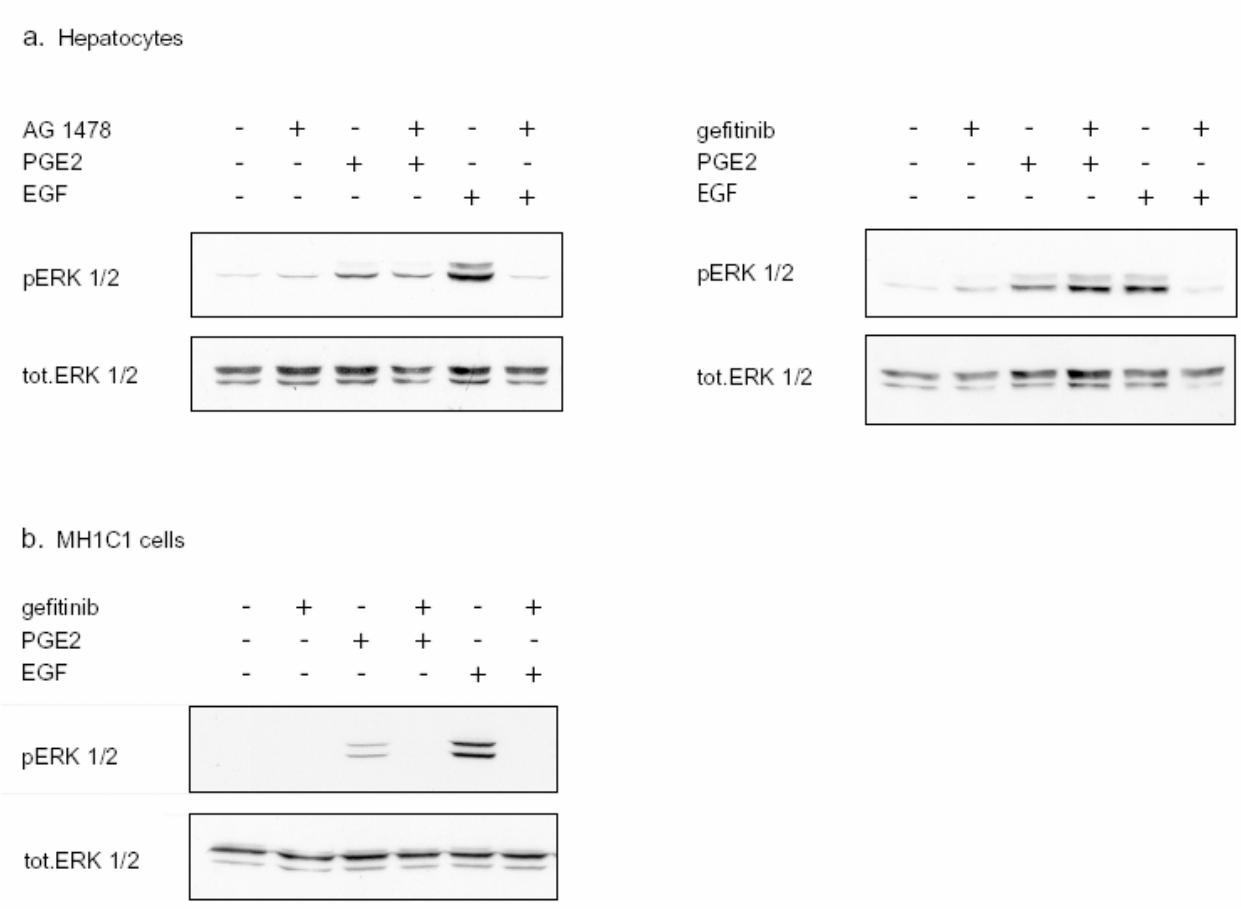
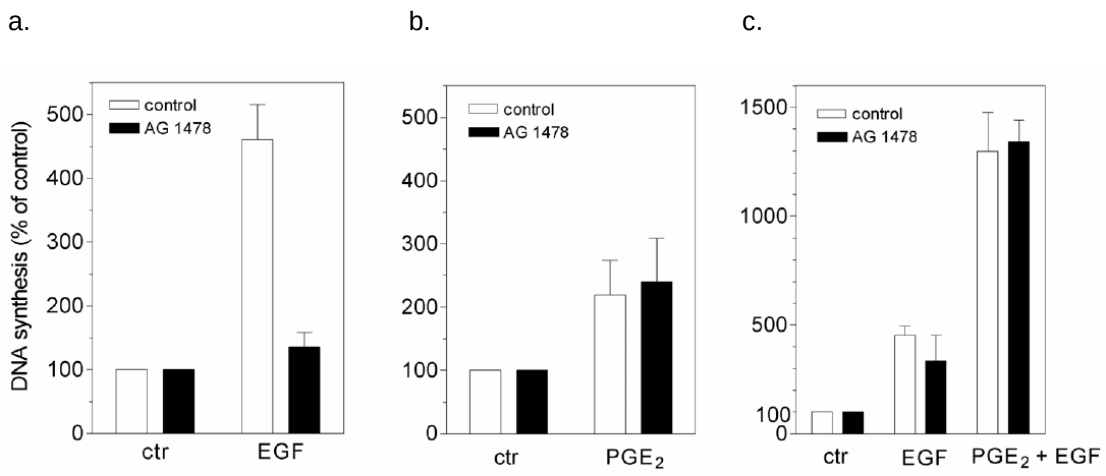


Figure 3 Transphosphorylation of the EGFR is necessary for the PGE2 induced activation of ERK in MH1C1 cells, but not in hepatocytes. Hepatocytes and MH1C1 cells were incubated with EGF (10 nM) or PGE2 (100 μM) for 5 min, after 3 h in culture. AG1478 (10 μM) or gefitinib (5 μM) was added 30 min before the addition of EGF or PGE2.

The comitogenic effect of PGE2 in hepatocytes is not dependent on transphosphorylation of the EGFR

Knowing that PGE2 enhances EGF induced DNA synthesis we wanted to find out if this comitogenic effect is dependent on transactivation of the EGFR. Hepatocytes were stimulated with PGE2 in early G1 (at 3 h in culture) and EGF was added in mid/late G1 (at 24 h). AG1478 was added shortly before PGE2 (at 2,5 h in culture). As the effect of AG1478 is transient it was necessary to repeat the treatment with the inhibitor a while after the addition of PGE2 (at 7 h in culture). The effect of AG1478 on cells treated with PGE2 alone was examined in an equivalent set-up without the addition of EGF. The results (fig. 4.) show that the addition of only PGE2 slightly increases DNA-synthesis, whereas PGE2 in combination with EGF synergistically increases the mitogenic effect of EGF. While AG1478 efficiently inhibits EGF induced DNA synthesis it affects neither the small mitogenic nor the comitogenic effect of PGE2. This indicates that transphosphorylation of the EGFR is not necessary for PGE2 to augment EGF induced mitogenesis.



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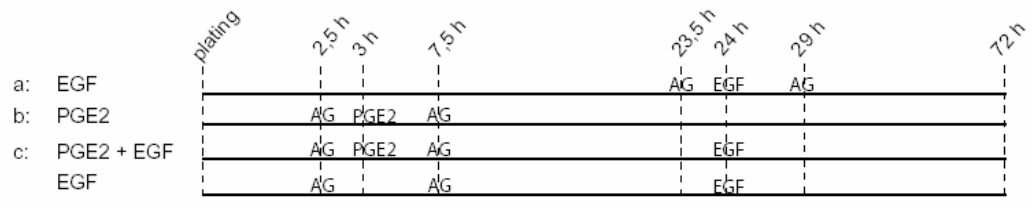


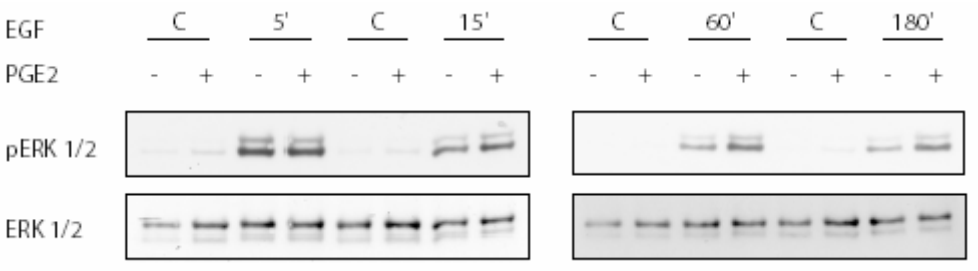
Figure 4 The comitogenic effect of PGE2 in hepatocytes is not dependent on transphosphorylation of the EGFR. **a.** EGF (10 nM) was added at 24 h and AG1478 (5 μM) at 23,5 and 29 h. The result confirms that inhibition of the EGFR by AG1478 is effective. **b.** PGE2 (100 μM) was added at 3 h and AG 1478 (5 μM) was added 2,5 h and 7,5 h. PGE2 has a slight mitogenic effect of its own, and it is not inhibited by AG1478. **c.** Hepatocytes were pretreated with PGE2 (100 μM) at 3 h and AG1478 (5 μM) at 2,5 and 7,5 h. EGF (10 nM) was added at 24 h. The comitogenic effect of PGE2 was not inhibited by AG1478. The same experiment was done without PGE2, i.e. AG1478 was added at 2,5 and 7,5 h, and EGF was added at 24 h. In this last experiment without PGE2 there is a slight inhibition of the mitogenic effect of EGF, due to the fact that the transitory effect of AG1478 is not completely reversed at the time of addition of EGF. All cells were harvested at 72 h. Figure a, b and c were borrowed from other members of the group. **d.** Time table for the addition of PGE2, EGF and AG1478 in figure 4 a, b and c.

Pretreatment with PGE2 enhances phosphorylation of ERK in hepatocytes after stimulation with EGF

As mentioned above, while ERK is essential to the mitogenic effect of EGF in hepatocytes, the comitogenic effect of PGE2 is not dependent on the activation of ERK in early G1. It is conceivable, though, that the transmission of the comitogenic signal involves ERK at a later stage in the cell cycle. We therefore examined the development of ERK phosphorylation after stimulation with EGF in hepatocytes that had been pretreated with PGE2 at an earlier stage. The set-up for the experiment was similar to the one illustrated in fig. 4c. PGE2 was added in early G1 (at 3 h) and EGF was added in mid-/late G1 (at 24 h). Phosphorylation of ERK was measured after 5, 15, 60 and 180 min. The results (fig. 5) show that pretreatment with PGE2 increases phosphorylation of ERK. This effect can be traced for as much as three hours after the addition of EGF. In other words, as PGE2 both increases EGF induced DNA synthesis and enhances EGF

induced ERK phosphorylation it is possible that the comitogenic effect of PGE2 is mediated through ERK.

a.



b.

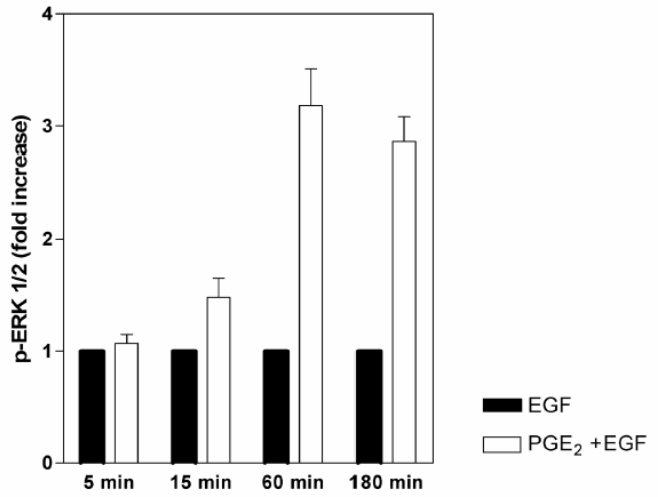


Figure 5 Pretreatment with PGE2 enhances phosphorylation of ERK in hepatocytes after stimulation with EGF. **a.** Hepatocytes were stimulated with PGE2 (100 μM) at 3 h in culture and EGF (10 nM) was added at 24 h. The cells were harvested 1 and 3 h after the addition of EGF. The enhancement of ERK phosphorylation is evident after 1 and 3 h. **b.** The graph shows fold increase of phosphorylated ERK in cells pretreated with PGE2 compared to cells treated with EGF only. The figure shows mean +/- SEM of 3 representative experiments.

Pretreatment with PGE2 imitates the effect of the protein phosphatase inhibitor vanadate on ERK phosphorylation in EGF stimulated hepatocytes

The presence of a substance that inhibits the dephosphorylation of phosphorylated ERK could produce results that resemble the ones presented in fig. 5. We therefore treated hepatocytes with vanadate, which inhibits the phosphatases that dephosphorylate activated ERK, i.e. the dual specifity phosphatases MKP1 and PAC1, and the nonreceptor phosphotyrosine phosphatases. PGE2 and EGF were added as done in the previous experiment (at 3 and 24 h, respectively), and vanadate was added 30 min before EGF. The results (fig. 6) show that pretreatment with PGE2 imitates the effect of vanadate. In cells stimulated with EGF only the amount of phosphorylated ERK declines after an hour. This decline is far less pronounced in cells treated with vanadate, and the same effect is seen in cells pretreated with PGE2. There is reason to assume that PGE2 and vanadate operate by the same mechanism since there is no additivity in cells that have been exposed to both substances in addition to EGF. These results support the suggestion that PGE2 may operate by inhibiting the dephosphorylation of activated ERK.

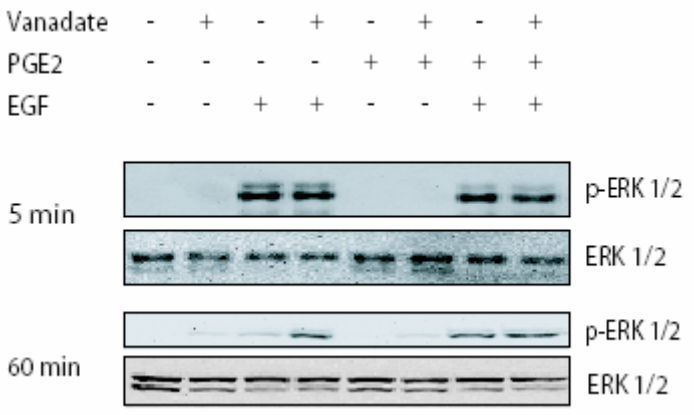


Figure 6 Pretreatment with PGE2 imitates the effect of the protein phosphatase inhibitor vanadate in EGF stimulated hepatocytes. PGE2 (100 μM) was added at 3h, EGF (10 nM) at 24 h, and vanadate at 23,5 h after plating. Cells were harvested 5 min and 60 min after the addition of EGF. The figure was borrowed from other members of our group.

Pretreatment with PGE2 imitates the effect of the protein synthesis inhibitors cycloheximide and actinomycin in EGF stimulated hepatocytes

PGE2 can phosphorylate ERK in hepatocytes after only 5 min (fig. 3), but it is evident from the results in fig. 5 that PGE2 also has another effect on ERK which takes hours to develop. This observation brings to mind the possibility that a more time consuming process such as protein synthesis may be involved in the transmission of the comitogenic signal from PGE2. To confirm this assumption we treated hepatocytes with inhibitors of protein synthesis. Actinomycin, which inhibits transcription, or cycloheximide, which inhibits protein synthesis by interfering with the translocation reaction on ribosomes, was added to the cells at 12 h before the cells were harvested. PGE2 and EGF were added at 3 h and 24 h respectively. The results (fig. 7) are similar to those from the experiment with vanadate: Pretreatment with actinomycin or cycloheximide prolongates the phosphorylation of ERK after stimulation with EGF. Pretreatment with PGE2 has a similar effect. There is no additivity between PGE2 and the protein synthesis inhibitors when the cells are treated with both prostaglandin, growth factor and protein synthesis inhibitor. (The only exception to this is found in cells treated with actinomycin at 180 min, where a small additive effect of PGE2 and actinomycin can be seen.) These results support the notion that the PGE2 may act indirectly, by altering gene transcription and inducing substances that inhibit the dephosphorylation of phosphorylated ERK.

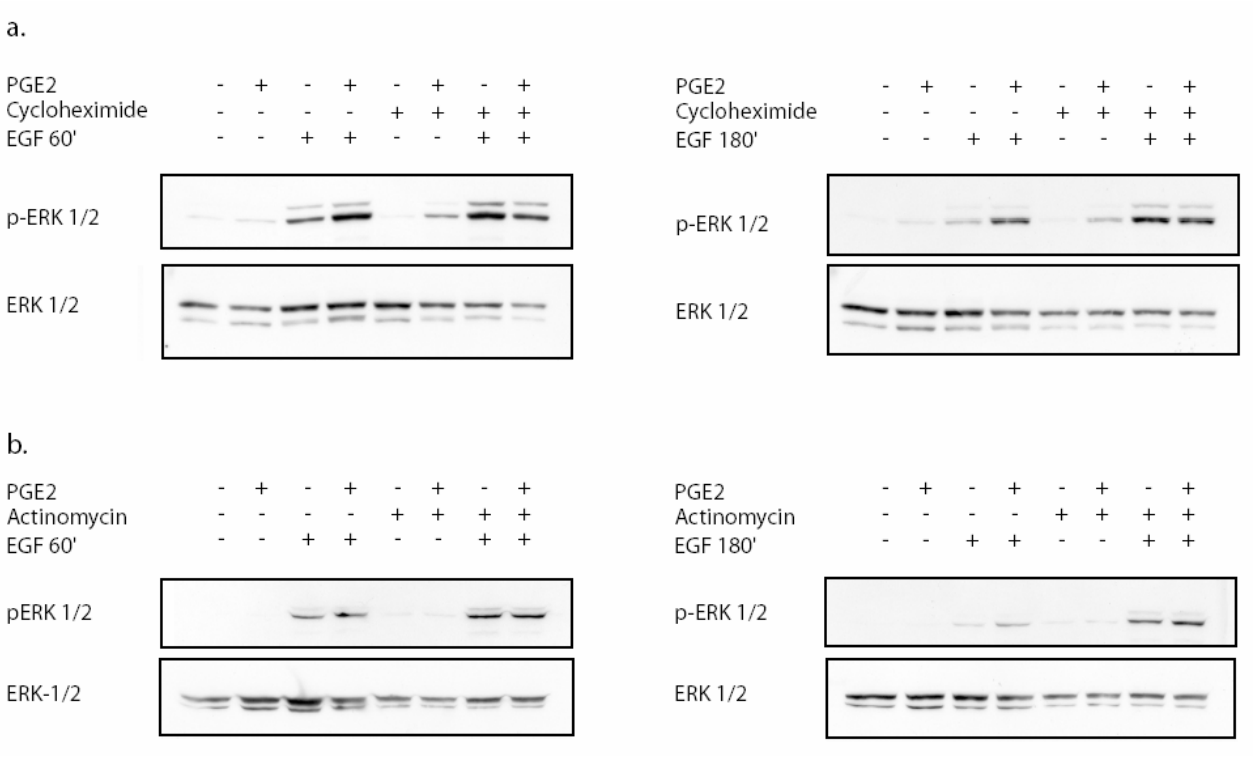


Figure 7 Pretreatment with PGE2 imitates the effect of the protein synthesis inhibitors cycloheximide and actinomycin in EGF stimulated hepatocytes. Hepatocytes were pretreated with PGE2 (100 μM) at 3 h and stimulated with EGF (10 nM) at 24 h in culture. ERK was measured 60 and 180 min after the addition of EGF. Cycloheximide (5 μg/ml) or actinomycin (0,2 μg/ml) was added 12 h before the cells were harvested.

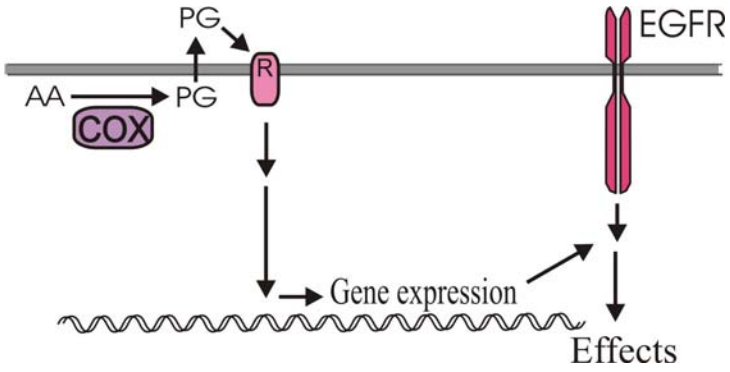


Figure 8 Prostaglandins modify gene expression in hepatocytes and affect signalling downstream of the EGFR. The figure was borrowed from other members of our group.

Conclusion

In the present study we have shown that PGE2 enhances the mitogenic effect of EGF on hepatocytes, and we have suggested a mechanism for the cross talk between the pathways activated by PGE2 and EGF in hepatocytes. Transactivation, which can explain cross talk in many cells lines, including the hepatoma cell line used in this study, is not involved in the hepatocytes we have studied. The fact that PGE2 has a long term effect on certain components in the EGFR pathway in hepatocytes is compatible with the notion of protein synthesis as a mechanism involved in the cross talk. This suggestion is supported by the fact that PGE2 pretreatment of hepatocytes imitates the effect protein synthesis inhibitors have on ERK activation upon stimulation with EGF. Pretreatment with PGE2 also imitates the effect of phosphatase inhibitors in an equivalent set-up. We therefore suggest that PGE2 interacts with the Ras/MAPK signalling pathway in an indirect way, by modulating gene transcription and inducing the synthesis of substances that inhibit the phosphatases that normally dephosphorylate activated ERK. More research is needed on this issue.

ABBREVIATIONS

AP-1	activator protein 1
c-Fos	The term fos was first used for the oncogene encoded by *F*inkel-Biskis-Jinkins murine *o*steogenicsarcoma virus. c-Fos is the normal genetic sequence (the proto-oncogene) from which the viral oncogene was derived.
CKI	Cdk inhibitor protein
COX	cyclooxygenase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erb B	receptor tyrosin kinase encoded by retrovirus-associated DNA sequences (erbB) originally isolated from, or related to, the avian erythroblastosis virus (AEV)
ERK	extracellular-regulated kinase
Ets	E twenty-six domain transcription factors
GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK 1/2	MAP/ERK kinase
MKP1/2	MAPK phosphatase
PGE2	prostaglandin E2
PGF2α	prostaglandin F2α
PI3K	phosphatidylinositol 3-kinase
PH domains	Pleckstrin homology domain
PP2A	protein phosphatase 2A
PTB domains	phosphotyrosine binding domain
RTK	receptor tyrosine kinase
SH2/SH3 domains	src homology 2/3 domain
Shc	src homology 2 domain-containing protein
Sos	son of sevenless
src	src is the cellular counterpart of v-src, which is the transforming product of Rous sarcoma virus.
TGFα	transforming growth factor α

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